Enhanced production of bioactive compound in the callus culture of *Glycyrrhiza glabra* L. with the improved biomass accumulation

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Original Article

Abstract

Licorice (Glycyrrhiza glabra) of Fabaceae family is known to have wide range of medicinal properties due to the metabolites found in the plant's tissue. Callus cultures from Glycyrrhiza glabra were previously initiated in vitro using leaf as an explant. The current study was designed in a way to examine the possible role of different elicitors concentrations on the stimulation of biomass and their effects on different metabolite content such as total carbohydrates, protein, proline, phenol, alkaloid, flavonoid and glycyrrhizin. Elicitation with different concentration of elicitors increased the biomass and metabolite content in callus culture of G. glabra at different rates. The optimum concentration of adenine sulphate for maximum biomass accumulation (16.79 g/flask) was found to be at 50 mg/l on the incubation of 20 th days. Adenine sulphate as well as putrescine was also found to stimulate the different metabolite content to 3-4 folds in the callus cultures as compared to that of control. Results showed that the metabolite content and antioxidant enzyme of Glycyrrhiza glabra can be enhanced by appropriate forms of elicitation.

Key Message

The metabolite content and antioxidant enzyme of *Glycyrrhiza glabra* was enhanced by elicitation. This provides opportunity for intensive research in cell exploitation for increasing metabolites, investigation of defense mechanism and regulation of metabolism.

Introduction

Glycyrrhiza glabra (licorice), a perennial herb of Fabaceae family, is abundantly rich in ameliorating bioactive secondary metabolites used for the treatment of a wide range of human ailments (Wang *et al.* 2013). The major constituents of licorice are triterpenoids and flavonoids. A variety of additional constituents such as triterpene, saponins, flavonoids, polysaccharides, pectins, simple sugars, amino acids, mineral salts, starch, gums, mucilage, essential oil, fat, tannins, glycosides, protein, resins, sterols, volatile oils and various other compounds have also been derived from licorice (Blumenthal *et al.* 2000).

Glycyrrhizin (potassium-calcium-magnesium salts of glycyrrhizic acid), a triterpenoid, is the most active constituent which reflects the sweet taste of licorice root. It is exclusively obtained from the dried roots and stolons of licorice and used in the treatment of liver and allergic diseases. Glycyrrhizin is 50 times or more sweeter than sugar and demands high prices globally as a non-nutritive sweetener (Jaiswal *et al.* 2017). It is manufactured in the form of injections (such as Stronger Neo-Minophagen®C) and tablets (such as Glycyron®) which are available in India and many other countries (Hayashi and Sudo 2009). Gels containing glycyrrhizin are used for the treatment of oral diseases and genital lesions caused due to *Herpes simplex* virus (Varsha *et al.* 2009).

Attempts were made to understand the biochemistry of licorice and its derivatives. Glycyrrhizin are synthesized through their specific metabolite pathways and possess specific structural and functional characteristics. Biochemical synthesis of the metabolite for industrial use is often not feasible due to complex metabolic pathways, complicated structures and chirality exhibited by these compounds (Namdeo 2007). The commercial demand can only be met by obtaining them directly from field grown plants. However, *G. glabra* accumulates glycyrrhizin in small amounts in specialized tissues probably after attaining a certain stage in their life cycle. Apart from this, the yields from field grown plants are influenced by many factors like climate, pests and diseases which are difficult to control and in turn affect their consistent production, due to which the commercial exploitation becomes a challenging task (Oksman-Caldenteyl and Inze 2004).

Recent developments in plant tissue culture techniques alongwith their processing have shown promising results to improve the productivity to many folds and have made it possible to gradually replace the whole plant cultivation as a source of useful secondary metabolites. Today, various tissue culture techniques are being used to enhance yield of secondary metabolites by invigorating plant defense and triggering stress response in plant cells with the help of elicitors (Chattopadhyay *et al.* 2002). Elicitors are being used as an enhancement strategy in plant secondary metabolite synthesis as they play an important role in stimulating the biosynthetic pathways leading to enhanced production of commercially important compounds. This provides an opportunity for intensive research in the field of plant sciences not only to exploit plant cells for increased yield of secondary metabolites, but also for the investigation of plant defense mechanism and regulation of secondary metabolism (Radman *et al.* 2003).

Materials And Methods

Plant material

Cuttings of *Glycyrrhiza glabra* were procured from the nursery of Central Institute of Medicinal Aromatic Plants (CIMAP), Lucknow, and maintained in the greenhouse (relative humidity 70-90%; temperature 25-30°C). The stem and leaves were excised and washed under running tap water for 20-30 minutes to remove the contaminants. Explants were then surface sterilized with 2-3 drops of Tween 20 and 0.1% mercuric chloride for 5-10 minutes followed by repeated rinses with sterile distilled water prior to *in vitro* culture.

Establishment of callus culture and culture conditions

Callus induction was initiated from the stem segments and young leaves of one-month old plants of licorice, sterilized as above and then gently cut or scraped using a scalpel. The explants were then inoculated on MS medium **(Murashige and Skoog 1962)** supplemented with the optimized combinations of phytohormones BAP (2.0 mg/l) and 2,4-D (0.5 mg/l) along with 3% sucrose and 0.8% agar, as reported by **Jaiswal *et al*. (2017)**. The medium was adjusted to pH 5.8 and autoclaved at 15 psi for 15 minutes. After inoculation the cultures were incubated at $25\pm2°C$ under at photoperiod of 12 hours with a light intensity of 40 μmolm^{-2}s^{-1} provided from cool white fluorescent tubes. Callus thus formed was sub-cultured every 30 days on the same, fresh medium.

Elicitation

The effect on plant growth and biochemical metabolites of various abiotic elicitors including adenine sulphate, biotin, salicylic acid and polyamines (putrescine, spermine and spermidine) at three different concentrations (30, 60 and 90 mg/l) was studied. All elicitors above were prepared as a stock solution and then added to the callusing media.

Estimation of primary and secondary metabolites

The Anthrone method was used for total carbohydrates determination **(Hedge and Hofreiter 1962)** and the results were expressed as glucose in milligram per gram of sample. Total protein was determined according to the method given by **Lowry *et al*. (1951)** and recorded as bovine albumin serum in milligram per gram of sample. Total phenol was assessed according to the method by **Bray and Thorpe (1954)** with results expressed as catechol in milligram per gram of sample. Proline was determined according to the method given by **Bates *et al*. (1973)**. Total alkaloid, expressed in milligram per gram of sample, was measured according to **Fazel *et al*. (2008)** using atropine as a standard. Total flavonoid was evaluated according to the method of **Chang *et al*. (2002)** using quercertin as a standard and expressed in milligram per gram of sample.

Glycyrrhizin estimation

HPLC analysis of glycyrrhizin was carried out using the method by **Hurst *et al*. (1983)**. The callus as well as the leaves and roots of field grown plants were weighed, crushed and extracted using 50% methanol under ultra-sonication (Elma, Germany), for 30 mins at 45 MHz. The extract was filtered (Millipore filter 0.45 μm) and concentrated on a rotary-evaporator (Buchi, Switzerland) and 2 ml of solvent was added to the sample before analysis. The resultant extracts were then used for subsequent HPLC analysis. A 10μl aliquot of extract was analysed by HPLC at 30°C. The HPLC system consisted of Waters HPLC 510 pump, a Nova-pak C18 column (3.9 × 150 mm, Waters, United States), a Waters 2478 detector, and a Millennium chromatography data system (Waters). The separation was performed with an isocratic elution using methanol–water–acetic acid (60: 34: 6) at a flow rate of 1 ml/min with UV absorption detection at 254 nm. Routine sample calculations were made by comparison of the peak area with that of the standard.

Assay of antioxidant enzyme activity

Approximately 0.5 g fresh samples were homogenized in 8 ml of 50 mM PBS (pH 7.6) including 0.1 mM Na-EDTA, and then centrifuged for 15 mins at 20,000 rpm and 4°C. The superoxide dismutase (SOD) activity was estimated by recording the decrease in optical density of formazone made by superoxide radicals and nitroblue tetrazolium chloride (NBT) dye by the enzyme **(Cakmak and Marschner 1992)**. One unit of SOD activity was defined as the amount of enzyme required to cause 50% inhibition of the rate of NBT reduction measured at 560 nm. Peroxidase (POX) activity was measured as described by **Castillo *et al*. (1984)** and the enzyme activity was calculated as per extinction coefficient of its oxidation product, tetra-guaiacol. The activity of ascorbate peroxidase

(APX) was measured according to **Cakmak (1994)** and measured by monitoring the rate of H_2O_2-dependent oxidation of ascorbic acid.

Results

Effect of various elicitor concentrations on biomass accumulation

After 15 days callus were sub-cultured on to the best callusing media (MS + 2mg/l BAP + 0.5mg/l 2,4-D) fortified with the various elicitors (biotin, adenine sulphate, salicylic acid, putrescine, spermine or spermidine) at different concentrations to optimize biomass accumulation. Biomass accumulation (25, 50, 75 and 100 mg/l) was recorded for different time intervals (10, 20 and 30 days) as shown in Table 1.

Biomass accumulation in control was 3.15, 5.66 and 8.21 g/flask after 10, 20 and 30 days of incubation respectively. Elicitation with different elicitors increased the biomass in callus culture of *G. glabra* at different, significantly different rates (Table 1). The optimum concentration of biotin (B), salicylic acid (SA) and spermidine (SD) was 75 mg/l, whereas that of adenine sulphate (AdS), putrescine (P) and spermine (SP) was 50 mg/l after incubation for 20 days.

Maximum biomass production was found with adenine sulphate (16.79 g/flask) elicitation followed by putrescine (14.23 g/flask) at 50 mg/l. Elicitation fostered, a 2-6 fold increase in biomass as compared to the control culture. Biomass production (callus weight) varies during incubation i.e., first it increases and reaches its peak on the 20th day and then it abruptly begins to decline.

The change in biomass production induced by various elicitors was found to be dependent on their concentration as well as on the incubation time. The biomass accumulation was affected by high concentration of elicitors (75-100 mg/l) in the medium as well as by prolonged incubation period (beyond 20 days) resulting into cell browning with decreased viability and cell death. Thus, increased callus growth in *G. glabra* is triggered when an adequate concentration of the appropriate elicitor is incorporated in the medium and callus are incubated for a specified period.

Effect of various elicitors on the production of different biochemical metabolites

The effect of the exogenous elicitors on the variation of various phytoconstituents present in licorice was evaluated and is summarized in Table 2 and Table 3. The concentration at which elicitors (biotin, adenine sulphate, salicylic acid, putrescine, spermine and spermidine) were showing highest biomass accumulation was selected for further biochemical analysis. The time of incubation of callus with the elicitors was of 20 days.

Elicitation seems to enhance the production of different metabolites in *G. glabra* at different rates (Table 2 and Table 3). The elicitors applied chemical stress to the callus culture triggering the production of different primary and secondary metabolites at rates that are normally not produced. Seven different biochemical parameters viz. carbohydrates, proteins, proline, phenols, alkaloids, flavonoids and glycyrrhizin were noted to be higher in the callus culture as compared to the leaves and roots of *in vivo* plants. Callus treated with the elicitors also showed higher metabolite content than the untreated callus.

Among the different elicitors, adenine sulphate and putrescine proved to be most effective in enhancing primary metabolite content in callus culture of licorice. The callus treated with 50 mg/l putrescine showed the highest carbohydrates content (54.82 mg/g) followed by those treated with 50 mg/l adenine sulphate (49.71 mg/g) whereas the highest content of protein (28.41 mg/g), phenol (36.46 mg/g) and proline (0.082 μmol/g) were found in callus treated with 50 mg/l adenine sulphate. The lowest metabolite content was found in callus treated with 75 mg/l spermidine. (Fig. 1)

Similarly, among the different elicitors tried, putrescine (75 mg/l) gave the highest alkaloid content (13.71 mg/g) whereas adenine sulphate (75 mg/l) showed the highest flavonoid content (16.29 mg/g). The least alkaloid and flavonoid content (4.69 and 7.12 mg/g) were found in spermidine treated callus. Glycyrrhizin was absent in the leaves of the field grown plant while glycyrrhizin content was highest in adenine sulphate treated callus (35.44μg/g) and lowest (8.02 μg/g) in untreated callus. (Fig.2)

It may thus be assumed that the *in vitro* raised tissues have higher content of metabolites than the *in vivo* plants and that these are enhanced by all the elicitors although at different concentrations and varying rates. Also, the callus treated with elicitors had higher

metabolite content than the untreated callus (control).

Effect of various elicitors on the activity of antioxidant enzyme

The activities of superoxide dismutase (SOD), ascorbate peroxidase (APX) and peroxidase (POD) have been analyzed to gain some insights of the cellular antioxidant responses triggered by elicitation. The antioxidant enzyme activities of callus increased rapidly after elicitation (Table 4). The enzyme activities of calluses treated with elicitors were found to be higher than that of leaves, roots and control callus. Superoxide dismutase (1.382 unit/mg protein) and ascorbate peroxidase (0.531 unit/mg protein) activity was maximum in callus treated with adenine sulphate while peroxidase activity (0.733 unit/mg protein) was highest in putrescine treated callus.

Discussion

Elicitation studies in callus culture using a wide range of elicitors have been documented to be successful in boosting the accumulation of biomass and metabolite production (Ram *et al.* 2013). During elicitation the optimal age of the culture varies between different plant cell system (Ahmed *et al.* 2014). The reaction of the cells to elicitor exposure depends on the growth stage of the culture thereby influencing the production of biomass and secondary metabolite (Deepthi and Satheeskumar 2016).

The efficiency of elicitors on cell growth and product yield varied according to their concentration, genotype, incubation period and culture characteristic, which may be due to the variations between different species, different cell lines and different cell physiology (Zhao *et al.* 2010). Introduction of elicitors changes the product aggregation pattern with the incubation time (Ahmed *et al.* 2014). Browning, retardation of growth and decrease in viability by inhibiting cell division due to increased concentration of elicitors were reported in various cultures (Deepthi and Satheeshkumar 2016) and could be attributed to an excess of stress ions which induces osmotic imbalance resulting in reduced growth as recorded in other plants examined (Elmaghrabi *et al.* 2013).

The increase in the content of biochemicals of *in vitro* regenerated plants may be due to the effect of different phytohormones on *in vitro* raised plants (Mohapatra *et al.* 2008). Yadav and Singh (2012) also observed significantly higher chlorophyll, total sugars, reducing sugars and protein content for *in vitro* regenerated plants compared to mother plants of *Glycyrrhiza glabra*. The incorporation of increasing concentration of biotic and abiotic elicitors in the medium also resulted in higher carbohydrate, protein, flavonoid and phenol accumulation than *in vivo* and untreated *in vitro* raised plants of *M. quadrifolia* (Manjula and Mythili *et al.* 2012).

The adenine component of adenine sulphate (AdS) enhances the cell growth and shoot proliferation (Murashige 1974) as a cytokinin coadjuvant and is thus added to the culture media to promote growth as well as to reinforce the reaction usually attributed to cytokinin in terms of axillary and adventitious shoot proliferation alongwith caulogenesis and somatic embryogenesis (Van Staden *et al.* 2008; Gatica *et al.* 2010). AdS was identified to be most efficient in inducing callus of several other plant species, i.e. *P. dactylifera* (Sane *et al.* 2012), *R. hybrid* (Ram *et al.* 2015) and *D. hamiltonii* (Zang *et al.* 2016). Adenine sulphate as nitrogen source can be drawn more rapidly by the cell than the inorganic nitrogen (Singh and Patel 2014). Nitrogen is essential for plants and can influence various physiological processes that regulate growth and morphogenesis according to its availability, source and concentration (Ramage and Williams 2002). Chandler and Dodds (1983) noticed that nitrogen influenced *in vitro* production of secondary products of *S. laciniatum*. Adenine sulphate (a nucleoside base) assist sulphate assimilation and amino acid biosynthesis necessary for the synthesis of metabolites (Sharma *et al.* 2014). In a variety of cultures, it serves as a cytokine synergist, a substrate for cytokinin synthesis and may delay cytokinin degradation by feed-back inhibition, or by competing for cytokinins metabolism enzymes (Van Staden *et al.* 2008). Adenine sulphate was found to enhance the different metabolite contents in several other plant species including *C. asiatica* L. (Sharma *et al.* 2014), *T. grandis* L. (Akram and Aftab 2015), *W. somnifera* L. (Sivanandhan *et al.* 2015)

In conjugation with other media components, the exogenous provision of vitamins has been documented to have a direct and indirect impact on different processes, such as callus growth, embryonic development, rooting and somatic growth (Abrahamian and Kantharajab 2011), acting as a cofactor of several enzymes considered essential for the metabolism of nutrients (Hildebrand *et al.* 2005). Biotin (water soluble B complex vitamin) is a source of nitrogen, and it manages and transport cytokinins needed for plant growth, development and metabolism. Being a heterocyclic compound, biotin serves as a cofactor for enzymes involved in

several reactions (carboxylation, decarboxylation, transcarboxylation and transamination) concerned with the fatty acid and carbohydrates metabolism and protein synthesis (Alban *et al.* 2000). Biotin promoted callus induction and proliferation in several other species such as *Capsicum annuum* (Kintzios *et al.* 2001), *Curcuma mangga* (Tamil *et al.* 2012) and *Phoenix dactylifera* (Diab 2015). However, no reports are available on metabolite production from callus culture treated with biotin.

Salicylic acid is extensively used to regulate the physiological, metabolic and biochemical activities of plants thereby affecting their growth (Orenes *et al.* 2013; Khan *et al.* 2015). SA stimulatory effects on callus and plant development have been reported in *C. officinalis* L. (Bayat *et al.* 2012), *Ziziphus spina christii* (Galal 2012) and *Vigna mungo* (Lingakumar *et al.* 2014). SA enhances both the primary as well as secondary metabolite in plants (Babel *et al.* 2014). It induces pathogen resistance protein and was successful in providing systemic acquired resistance to pathogens (George *et al.* 2008). SA potentially alters the metabolic pathways leading to the accumulation of phytoconstituents during *in vitro* culture (Ram *et al.* 2013), may be because SA directly or indirectly affects the synthesis and signalling pathways of auxins, ethylene and jasmonic acid (Vlot *et al.* 2009). Salicylic acid elicits different metabolites content in several plant species such as *Andrographis paniculata* L. (Zaheer and Giri 2015), *Bacopa monnieri* (Largia *et al.* 2015) and *Achillea millefolium* L. (Gorni and Pacheco 2016).

Polyamines (PAs) are small aliphatic amines (particularly, putrescine, spermine and spermidine) found in almost all plant cells (Sawhney *et al.* 2003). They engage in several stress reactions triggering cell division and differentiation (Liu *et al.* 2015), and could be an endogenous regulator for plant growth or hormonal messengers (Davis 2004). Several findings showed a strong association between polyamine level and several fundamental processes (cell division, tissue differentiation, organogenesis and embryogenesis alongwith macromolecular biosynthesis) (Aydin *et al.* 2016). Polyamines interact with phytohormones enhancing the production of callus in *M. charantia* (Thiruvengadam *et al.* 2012), *P. gerardiana* (Ravindra and Nataraja 2013) and *Phoenix dactylifera* (Ibrahim *et al.* 2014). PAs influence the membrane transport by regulating the proton pump which plays an important role in secondary metabolism (Janicka-Russak *et al.* 2010). PAs incorporation with phytohormones enhanced the secondary metabolite production in other species such as *P. corylifolia* L. (Shinde *et al.* 2009), *N. cataria* L. (Yang *et al.* 2010) and *P. ginseng* (Marsik *et al.* 2014).

Elicitors trigger the plant defense signals by an immediate cellular response which increases the accumulation of reactive oxygen species (ROS) including hydroxyl free radicals (OH^-), hydrogen peroxide (H_2O_2) and superoxide anions (O_2^-). In order to reduce these excessive ROS, different cell compartments use antioxidant enzymes like ascorbate peroxidase (APX), catalase (CAT), glutathione reductase (GR), peroxidase (POD) and superoxide dismutase (SOD) to activate the cell defensive systems (Martinez *et al.* 2016). Under light, chloroplast and peroxisomes are the main source of ROS production while in dark it is mitochondria (Foyer and Noctor 2005). The severe discrepancy between ROS production and antioxidant defenses leads to oxidative stress which causes cellular damages thereby increasing the production of secondary metabolites (Zhao *et al.* 2005). The application of elicitors induces ROS burst in the cultured cells of Parsley, *Taxus chinensis* and as well as in protoplasts of *Arabidopsis*(Wang and Wu 2005; Sasaki-Sekimoto *et al.* 2005). H_2O_2 enhanced the production of phytoalexin by activating the signaling defense genes and coordinating with antioxidant response (Ramos-Valdivia *et al.* 2012).

During the ROS detoxification process SOD catalyzed the primary reaction by providing first line of defense against the toxic effects of ROS (Ali *et al.* 2006), and abiotic elicitors have been shown to increase SOD, CAT, APX and POX activities (Martinez *et al.* 2016). Under unfavorable conditions SOD is the first to scavenge off toxic O_2^- level, and other antioxidant enzymes (APX, CAT, GR and POX) convert H_2O_2 into H_2O and molecular oxygen, preventing cellular damage (Scandalios 2005). In several plant genera, elicitation was found to increase stress which in turn resulted in an increased SOD activity (Samar *et al.* 2011). During elicitation, a similar pattern was observed for CAT, POD and APX activity, except for tissue and dose specific variation (Elkahoui *et al.* 2005).

SA induces the production of ROS thereby developing systemic acquired resistance (SAR) (Kawano and Muto 2000). Depending on the H_2O_2 concentration SA increases and reduces the activities of catalase (CAT) and peroxidase (POX) (Guan and Scandalios 2006). Defensive teams such as CAT and POX targeted protective cells from oxidative damage (Mittler 2002). SA enhanced the antioxidant activities in many other plant species (Rehman *et al.* 2017). Adenine sulphate was also reported to elicit different antioxidant enzyme activities in other plant species (Ahmad *et al.* 2017). Polyamines have effectively improved both enzymatic and non-enzymatic antioxidant activities (Asthir *et al.* 2012). Abundant studies have emphasized that the plants under stress showed an interaction between polyamines and reactive oxygen species (Pottosin *et al.* 2014).

Declarations

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Tables

Table 1. Effect of different elicitors on biomass accumulation of *in vitro* grown callus of *G. glabra* on MS media supplemented with 2 mg/l BAP, 0.5 mg/l 2,4- D and 40 mg/l Ascorbic acid

Elicitors conc. (mg/l)	Biomass Accumulation (g/flask)																	
	After 10 days						After 20 days						After 30 days					
	B	AdS	SA	P	SP	SD	B	AdS	SA	P	SP	SD	B	AdS	SA	P	SP	SD
25	4.18^c	4.89^c	4.48^c	6.99^{ab}	4.15^c	4.06^b	7.52^c	10.35^d	7.94^c	9.62^c	6.19^b	5.61^b	5.28^c	8.50^c	5.59^d	7.41^b	5.14^b	3.23^b
50	6.36^b	9.56^a	5.57^b	7.43^a	6.52^a	3.83^b	8.72^b	16.79^a	9.37^b	14.23^a	6.87^a	5.82^{ab}	6.71^b	12.37^a	6.73^b	10.61^a	6.02^b	3.36^a
75	6.99^a	6.35^b	7.14^a	6.41^b	5.22^b	4.95^a	11.02^a	13.91^b	11.64^a	10.74^b	6.24^b	6.37^a	8.94^a	9.92^b	9.83^a	7.18^b	4.72^a	4.34^a
100	4.44^c	5.13^c	4.65^c	5.24^c	4.11^c	3.10^c	6.92^d	11.04^c	7.89^c	8.03^d	4.92^c	4.57^c	5.01^c	8.28^c	6.00^c	6.00^c	4.24^c	2.23^c
G. Mean	5.49	6.48	5.46	6.52	5.00	3.98	8.55	13.02	9.21	10.66	6.05	5.59	6.48	9.77	7.04	7.80	5.03	3.29
S.E.M.	0.15	0.22	0.20	0.20	0.15	0.13	0.14	0.15	0.12	0.12	0.13	0.17	0.16	0.17	0.12	0.14	0.13	0.10
C.D. (5%)	0.49	0.71	0.65	0.64	0.49	0.42	0.45	0.49	0.39	0.40	0.41	0.57	0.54	0.56	0.40	0.44	0.42	0.32

All the value are calculated as mean. Means followed by different alphabet within a column are significantly different (p<0.05).

Table 2 Effect of different elicitors on the primary metabolites of *in vitro* grown callus of *G. glabra* on MS media supplemented with 2 mg/l BAP, 0.5 mg/l 2,4- D and 40 mg/l Ascorbic acid after 15 days

Elicitors	Carbohydrates (mg/g)	Protein (mg/g)	Phenol (mg/g)	Proline (μmol/g)
Leaves (field grown plant)	10.22^i	4.45^i	10.65^i	0.019^i
Root (field grown plant)	14.63^h	7.66^h	16.06^h	0.022^h
Control	19.74^g	11.41^g	20.53^g	0.043^g
Adenine sulphate (50 mg/l)	49.71^b	28.41^a	36.46^a	0.082^a
Biotin (75 mg/l)	38.79^d	20.52^d	28.44^d	0.063^d
Salicylic acid (75 mg/l)	45.99^c	24.38^c	33.35^c	0.070^c
Putrescine (50 mg/l)	54.82^a	26.35^b	34.10^b	0.076^b
Spermine (50 mg/l)	35.31^e	18.07^e	25.06^e	0.057^e
Spermidine (75 mg/l)	32.06^f	15.81^f	13.95^f	0.05^f
G. Mean	32.94	17.45	25.31	0.054
S.E.	0.26	0.33	0.20	0.001
C.D. (5%)	0.76	0.97	0.60	0.003

All the value are calculated as mean. Means followed by different alphabet within a column are significantly different (p<0.05).

Table 3 Effect of different elicitors on the secondary metabolites of *in vitro* grown callus of *G. glabra* on MS media supplemented with 2 mg/l BAP, 0.5 mg/l 2,4- D and 40 mg/l Ascorbic acid after 15 days

Elicitors	Alkaloid (mg/g)	Flavonoid (mg/g)	Glycyrrhizin (µg/g)
Leaves (field grown plant)	0.73[h]	3.99[h]	0.00[h]
Root (field grown plant)	1.89[g]	6.66[g]	8.02[g]
Callus (control)	3.47[f]	7.01[f]	9.55[f]
Adenine sulphate (75 mg/l)	11.51[b]	16.29[a]	35.44[a]
Biotin (50 mg/l)	6.78[d]	10.60[d]	11.46[d]
Salicylic acid (50 mg/l)	9.02[c]	13.95[c]	12.69[c]
Putrescine (75 mg/l)	13.71[a]	15.03[b]	15.03[b]
Spermine (50 mg/l)	6.49[d]	8.50[e]	10.47[e]
Spermidine (75 mg/l)	4.69[e]	7.12[f]	8.11[g]
G. Mean	6.48	9.90	12.31
S.E.	0.30	0.08	0.04
C.D. (5%)	0.90	0.23	0.10

All the value are calculated as mean. Means followed by different alphabet within a column are significantly different (p<0.05).

Table 4 Effect of different elicitors on the antioxidant enzyme activity of *in vitro* grown callus of *G. glabra* on MS media supplemented with 2 mg/l BAP, 0.5 mg/l 2,4-D and 40 mg/l Ascorbic acid after 15 days

Elicitors	SOD (unit/ mg protein)	APX (unit/ mg protein)	POD (unit/ mg protein)
Leaves (field grown plant)	0.913[h]	0.186[h]	0.337[g]
Root (field grown plant)	1.169[g]	0.338[g]	0.581[f]
Callus (control)	1.232[f]	0.360[f]	0.604[e]
Adenine sulphate (75 mg/l)	1.382[a]	0.531[a]	0.727[a]
Biotin (50 mg/l)	1.305[c]	0.434[c]	0.679[c]
Salicylic acid (50 mg/l)	1.316b[c]	0.453[b]	0.714[b]
Putrescine (75 mg/l)	1.323[b]	0.458[b]	0.733[a]
Spermine (50 mg/l)	1.286[d]	0.415[d]	0.673[c]
Spermidine (75 mg/l)	1.265[e]	0.379[e]	0.657[d]
G.Mean	1.24	0.40	0.64
S.E.	0.005	0.003	0.004
C.D. (5%)	0.02	0.10	0.01

All the value are calculated as mean. Means followed by different alphabet within a column are significantly different (p<0.05).
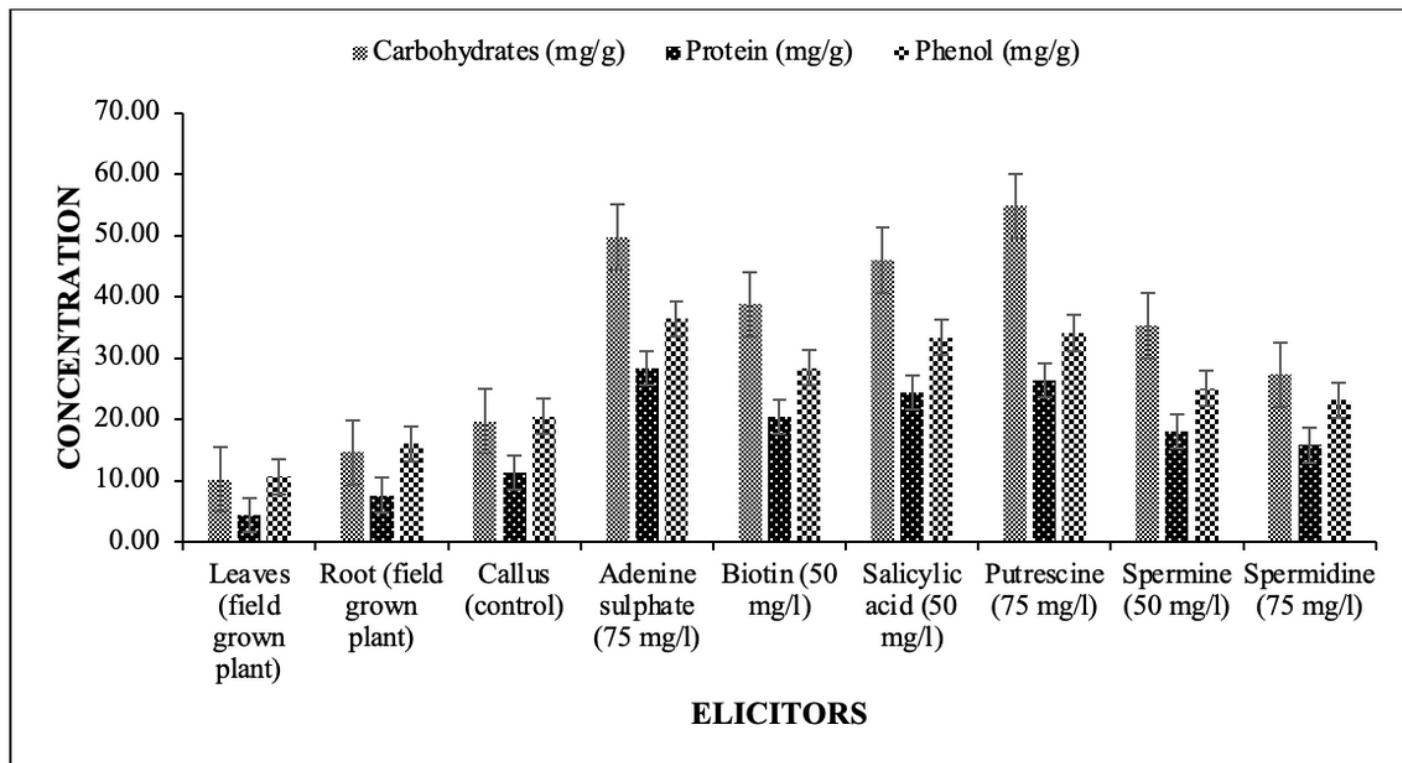
Figures

Figure 1

Effect of elicitors on primary metabolites of in vitro grown callus of G. glabra
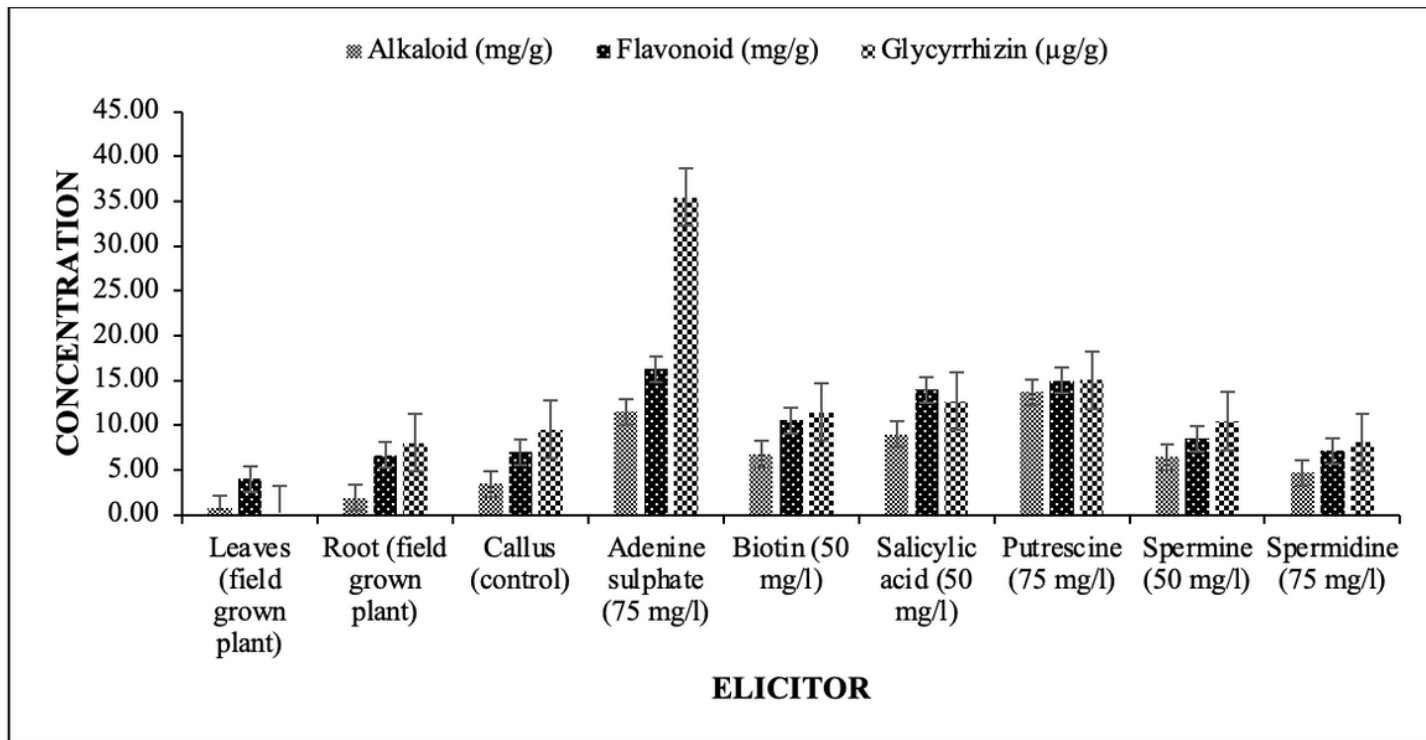

Figure 2

Effect of elicitors on secondary metabolites of in vitro grown callus of G. glabra
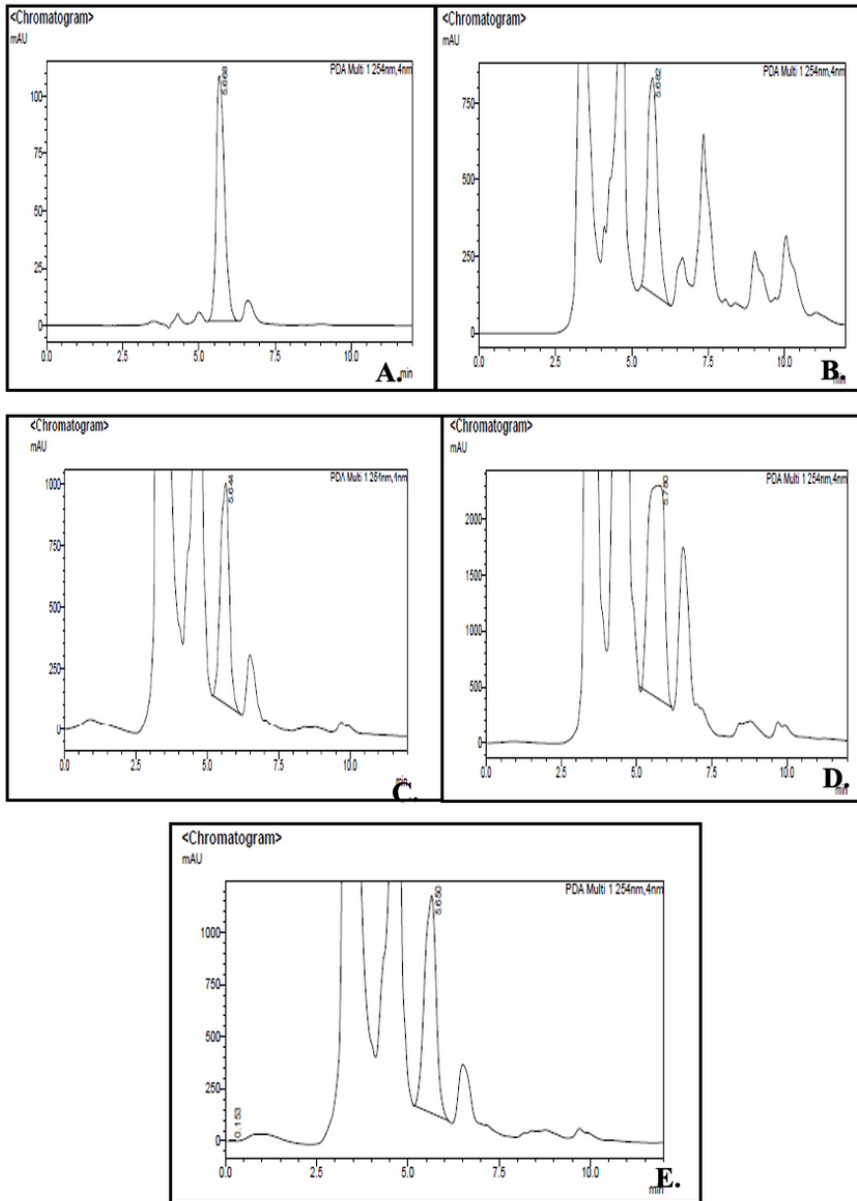
Figure 3

HPLC chromatogram for glycyrrhizin interpretation in the methanolic extract of root and callus (A). Standard (Pure glycyrrhizin) (B). Root (in vivo) (C). Callus without elicitor treatment (D). Callus treated with adenine sulphate (E). Callus treated with biotin
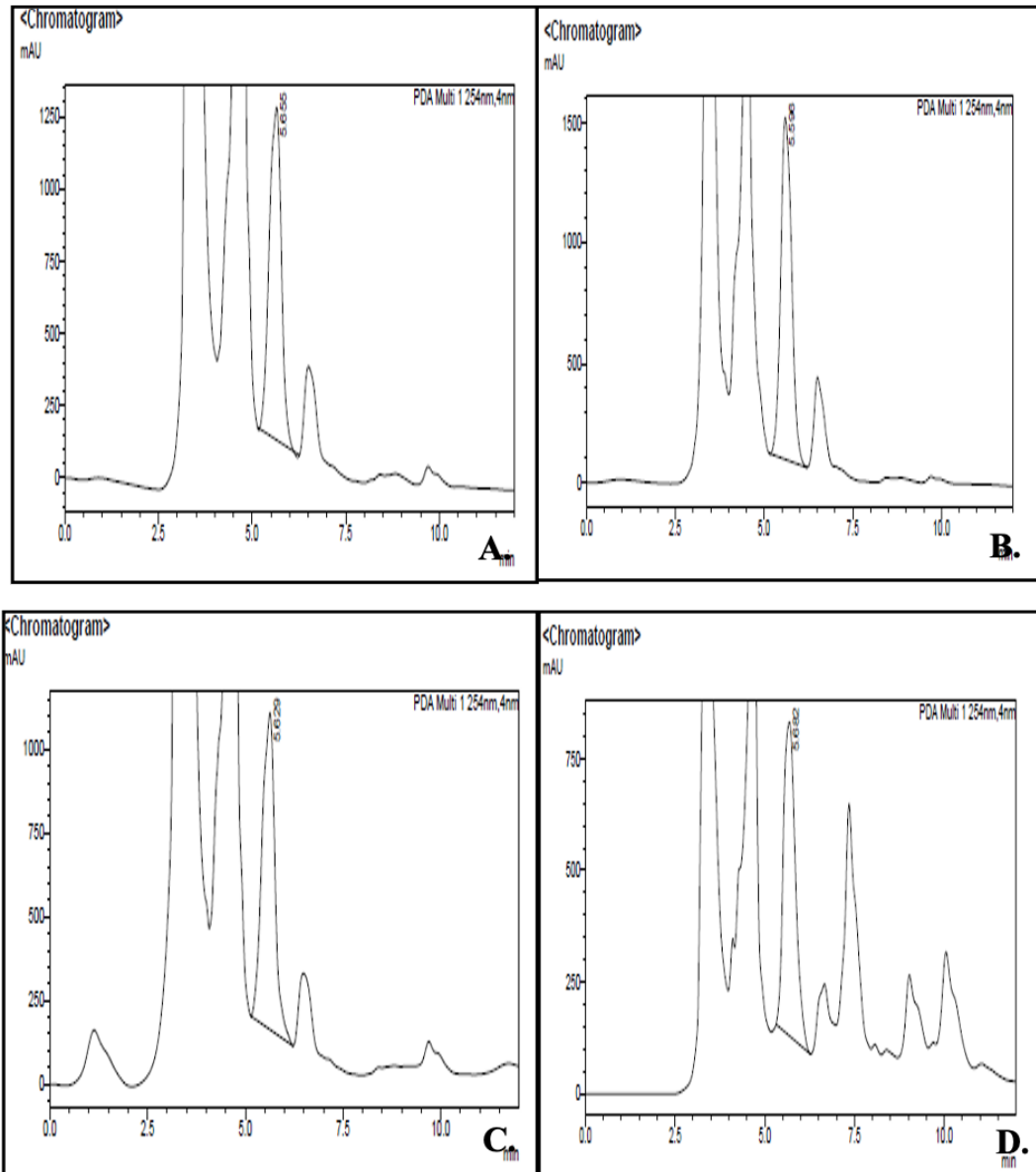
Figure 4

HPLC chromatogram for glycyrrhizin interpretation in the methanolic extract of callus (A). Callus treated with salicylic acid (B). Callus treated with putrescine (C). Callus treated with spermine (D). Callus treated with spermidine
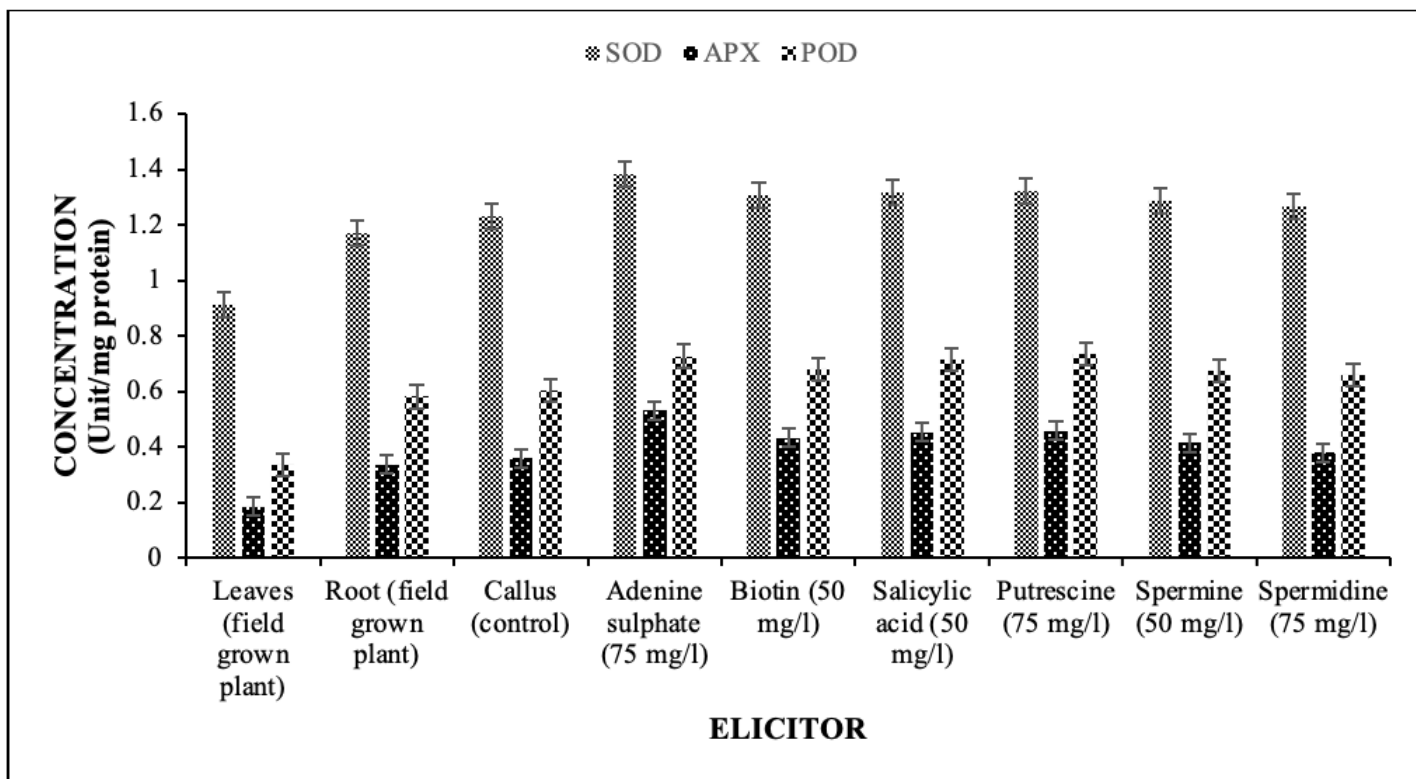
Figure 5

Effect of elicitors on antioxidant enzyme activity of in vitro grown callus of G. glabra